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GRANT NUMBER DAMD17-94-J-4050

TITLE: General Methods for Identifying G1-Phase Substrates of Cdk Protein Kinases

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REPORT DATE: June 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1996	Annual (1 Jun 95 - 31 May 96)

4. TITLE AND SUBTITLE
General Methods for Identifying G1-phase Substrates of Cdk Protein Kinases

5. FUNDING NUMBERS
DAMD17-94-J-4050

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We are using two-dimensional gels in combination with genetic and biochemical techniques to identify G1-phase substrates of cyclin-Cdk complexes. We have optimized protocols for labelling proteins with ^{32}P prior to two-dimensional gel electrophoresis. So far, we have successfully identified one important in vivo substrate, Sic1. We have characterized Sic1, and have shown that phosphorylation of Sic1 by cyclin-Cdk complexes promotes DNA synthesis in yeast. We have made peptide antigens for the purpose of making antibodies against phosphorylated Cdk substrates. Mice have been immunized; their sera are positive; and fusions for monoclonal antibodies are about to be done.

14. SUBJECT TERMS Breast Cancer

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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5. Introduction.

There is general agreement that in all eukaryotes, phosphorylation by various cyclin-Cdk complexes controls and orchestrates key cell cycle events. These events include commitment in G1 phase, initiation of DNA synthesis in S phase, and spindle formation and elongation in mitosis. However, despite knowing a great deal about the cyclin-Cdk complexes themselves, and despite years of investigation by many laboratories, we know only about half a dozen substrates of the cyclin-Cdk kinases, and none of these explain the control of critical cell cycle events. In particular, we do not know what substrates have to be phosphorylated for commitment to occur (although in mammalian cells, Rb is almost certainly one of the substrates).

The purpose of the present work is to develop methods for identifying substrates of the cyclin-Cdk complexes. In particular, we are interested in G1 substrates. To begin, experiments will be done in the yeast *S. cerevisiae*, and then the project will expand into mammalian cells.

We initially proposed two main approaches. The first approach uses two-dimensional gels to examine phosphoproteins. Various cyclins are expressed from a *GAL* promoter, and cells with the over-expressed cyclins are labelled with ^{32}P. The pattern of spots on a 2D gel is then compared between cells expressing and not expressing the cyclin. Extra spots in the cyclin-expressing cells may be substrates.

The second approach is to develop antibodies against phosphoserine followed by proline, and phosphothreonine followed by proline. Such antibodies would recognize proteins phosphorylated by Cdk complexes. Thus, such proteins could be immunoprecipitated and sequenced. This could also be combined with the 2D gels as an enrichment step.

6. Body of the Report.

A. Visualization of G1 Cdc28 substrates on 2D gels.

i. Optimize labeling, extraction, and gel conditions. Quite a number of experiments of this kind were done in the first year of the grant, and we optimized procedures for equilibrium 2D gels. In the year just past, we have extended this by optimizing conditions for non-equilibrium gels, which is important, because equilibrium gels were useful only for proteins with an iso-electric point of about 7.5 or less. It turns out that many potential substrates have isoelectric points much higher than this, and so need to be analysed on non-equilibrium gels. We are now able to do this.

ii. Identification of Sic1 as a Cln substrate. As proposed in the original grant, we induced *GAL-CLN1* and *GAL-CLN2*, labelled with 32P, and looked on 2D gels for spots appearing only when the G1 cyclins are induced. There are apparently three sets of weak spots that can be seen in many (but not all) experiments, possibly representing three substrates.

All of the spots were faint, probably representing very small amounts of protein. Methods were described in the original proposal for identifying low-abundance substrates. However, while beginning to apply these methods, we realized that from other work, we knew of proteins that might be substrates of Cdc28, and which in some cases had molecular weights and isoelectric points

roughly consistent with the phosphorylated spots we could see on the 2D gels. One of these was a protein called Mcm3, which is involved in DNA replication. Another is Cdc54, which is also involved in DNA replication. We are testing these proteins as in vivo substrates now. A third protein of interest was Sic1.

We showed that Sic1 could be phosphorylated in vitro by Cln-Cdc28 complexes. When Sic1 and cyclin-Cdc28 were mixed in a kinase reaction, and the products were run on 2D gels, we could see up to 13 charge isoforms of Sic1. We had some evidence that some of these also occured in vivo. We also showed that Sic1 was in fact a phosphoprotein in vivo, and that, as predicted, this phosphorylation depended on Clns.

With this preliminary evidence in hand, we went on to characterize the relationship of Sic1, Clns, and Start. This work has recently been published in Science (Schneider et al. 1996), and a reprint is attached.

We showed that Sic1 was a phosphoprotein in vivo. We showed that in vivo, hyper-phosphorylated forms of Sic1 depended on induction of Clns. We showed that degradation of Sic1 depended on the ubiquitin-conjugating enzyme Cdc34 (as had previously been shown by others), and also showed that degradation also depended on Clns. This was consistent with the idea that Cln-Cdc28 complexes directly phosphorylate Sic1, and that phosphorylated Sic1 is then degraded via Cdc34 and the ubiquitin pathway.

Genetic experiments supported this idea. Most strikingly, a *sic1* deletion suppressed the lethality of the *cln1 cln2 cln3* triple deletion strain. This suggests that an essential function of the Clns is promoting destruction of Sic1.

Sic1 is an inhibitor of Clb-Cdc28 complexes, and the earliest cell cycle roles of these kinases are spindle formation and initiation of S-phase. We demonstrated that in a *sic1* null mutant, S-phase was advanced with respect to budding, and the timing of S-phase was now almost independent of Cln expression. These two results suggest that S-phase is normally linked to Start via Sic1. In the absence of Sic1, S-phase becomes independent of Start and independent of Clns.

The Cln-Cdc28 complexes and the Clb-Cdc28 complexes are similar in many ways, and yet, as far as we know, only the Clb-Cdc28 complexes are inhibited by Sic1. Some biochemical experiments were done to extend our knowledge in this area. We found that all tested cyclin-Cdc28 complexes, including Cln1, Cln2, Cln3, Clb1, Clb2, and Clb5 complexes could phosphorylate Sic1 very well in vitro at low concentrations of Sic1. At higher concentrations of Sic1, the Clb1, Clb2, and Clb5 complexes were inhibited, but Cln1, Cln2, and Cln3 complexes were not. When cyclins were immunoprecipitated from such kinase reactions, substantial amounts of Sic1 co-precipitated with Clb1, Clb2, and Clb5, but little or no Sic1 co-precipitated with Cln1, Cln2 or Cln3. Thus, binding correlates with inhibition. It may be that Sic1 inhibits Clbs but not Clns because it binds tightly to Clbs (but not Clns) in the substrate binding cleft, and blocks the access of other substrates.

We tried similar experiments with p21, a human protein that inhibits human G1 cyclin/CDK complexes. Perhaps surprisingly, p21 was a specific inhibitor of Cln1, Cln2, and Cln3-Cdc28 complexes, but did not inhibit Clb1, Clb2, or Clb5 complexes (at least at the concentrations we tried). Once again, tight binding (as shown by co-immunoprecipitation) correlated with inhibition. It has been suggested that in human cells, p21 discriminates between different cyclin-CDK complexes by recognizing the CDK component. Our results with Cdc28 complexes show that it must also recognize the cyclin component, since the CDK was constant in our experiments.

B. Develop antibodies against phosphoSer-Pro and phosphoThr-Pro.

Because of the difficulty in just seeing spots phosphorylated by Cdc28 (not to mention the difficulty in purifying, identifying, and analysing them), we would like antibodies that could specifically recognize the phosphorylated forms of Cdc28 substrates. Since Cdc28 almost always phosphorylates a serine or threonine followed by a proline (i.e., SP or TP) we would like antibodies directed against phospho-S-P and phospho-T-P. It is likely that such antibodies can be generated; good antibodies have been made against phospho-tyrosine (Ross et al. 1981), and some antibodies have been made against phospho-threonine (Heffetz et al. 1989). The extra proline should make for a much better epitope than phospho-Ser or phospho-Thr alone.

In the past year, we tried to use the monoclonal antibody MPM2 to detect these kinds of phosphorylated proteins. These efforts were unsuccessful; we're not sure why.

We have synthesized a set of peptides to try and make the antibodies desired. The peptides are:

1. NH_2 C G G **pS** P G G K-OH COOH

2. NH_2 R A A **pS** P A A C-OH COOH

3. NH_2 C N N **pS** P N N H-OH COOH

4. NH_2 C G G **pT** P G G K-OH COOH

5. NH_2 R A A **pT** P A A C-OH COOH

6. NH_2 C N N **pT** P N N H-OH COOH

Each peptide has been conjugated (through the terminal cys residue) to three different carrier proteins, KLH, ovalbumin, and BSA. The immunization strategy to get anti-phospho-Ser-Pro antibodies has been to do a cycle of immunizations: peptide 1 coupled to KLH was the primary antigen; peptide 2 coupled to BSA was the first boost; peptide 3 coupled to KLH was the second boost; peptide 1 coupled to BSA was the third boost; peptide 2 coupled to KLH was the fourth boost; peptide 3 coupled to BSA was the fifth boost; and finally peptide 1 coupled to KLH was the sixth boost. Note that because of the different peptide sequences, the only thing in common between all the antigens was phospho-Ser-Pro. The sera have just been tested against the three peptides coupled to ovalbumin, which of course was not used for immunization. All sera are very strongly positive.

The phospho-thr-pro antigens have been treated identically, and the sera for these are also strongly positive.

We hope to do the fusions in the next few weeks, as soon as our monoclonal facility has time and space.

C. Future plans.

Identification and analysis of Sic1 as a substrate of G1 cyclins at Start took most of our effort in the past year. Much of that analysis is now done, and we will return to the task of finding more substrates. In particular, we will begin to examine spots seen on non-equilibrium gels at isoelectric points greater than 7.5.

It has become clear that visualization, identification, and analysis of in vivo labelled spots is very difficult. The method we used to identify Sic1--educated guessing--is even more powerful now that the full sequence of the genome is known, and we are applying it to additional spots. Also, anti-phospho-Ser-Pro and phospho-Thr-Pro antibodies may be very useful.

7. Conclusions.

The project is proceeding roughly as planned. We have probably spent more time than anticipated analyzing the one substrate we have found, but this extra analysis was worth the effort, and we are actually very happy to have even one substrate. The difficulty in visualizing and analyzing non-abundant spots was probably under-estimated in the original proposal, but we have developed and are continuing to develop methods to help us with this problem.

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Ross, A. H., Baltimore, D., and Eisen, H. (1981) Nature 294, 654.
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Reprint Series
26 April 1996, Volume 272, pp. 560–562

SCIENCE

Linkage of Replication to Start by the Cdk Inhibitor Sic1

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Linkage of Replication to Start by the Cdk Inhibitor Sic1

B. L. Schneider,* Q.-H. Yang,* A. B. Futcher†

In *Saccharomyces cerevisiae*, three G_1 cyclins (Clns) are important for Start, the event committing cells to division. Sic1, an inhibitor of Clb-Cdc28 kinases, became phosphorylated at Start, and this phosphorylation depended on the activity of Clns. Sic1 was subsequently lost, which depended on the activity of Clns and the ubiquitin-conjugating enzyme Cdc34. Inactivation of Sic1 was the only nonredundant essential function of Clns, because a *sic1* deletion rescued the inviability of the *cln1 cln2 cln3* triple mutant. In *sic1* mutants, DNA replication became uncoupled from budding. Thus, Sic1 may be a substrate of Cln-Cdc28 complexes, and phosphorylation and proteolysis of Sic1 may regulate commitment to replication at Start.

Before yeast can replicate DNA, they must pass Start, which requires a cyclin-dependent protein kinase composed of a catalytic subunit (Cdc28) and one of three G_1 cyclins (Cln1, -2, or -3) (*1*). After Start, B-type cyclin-Cdc28 kinases such as Clb5-Cdc28 and Clb6-Cdc28 must be activated to allow replication (*2*). Although Clb5- and Clb6-Cdc28 complexes are present in G_1 phase, they are initially inactive because of inhibition by the Sic1 protein (*2*, *3*). Activation of Clb5- and Clb6-Cdc28 occurs after Sic1 is targeted for proteolysis by the ubiquitin-conjugating enzyme Cdc34 (*2*). Thus, a *cdc34* mutant arrests with a 1N DNA content because it cannot degrade Sic1, but nevertheless buds, and duplicates its spindle pole body.

It is not known how Start triggers Sic1 inactivation or how replication is tied to other Start-dependent events such as budding and duplication of the spindle pole body. Is Start a single event that affects multiple pathways, or is Start a collection of events, one of which regulates Sic1 proteolysis and replication?

We asked whether Cln-Cdc28 complexes phosphorylate Sic1, thereby targeting it for proteolysis. Sic1 coprecipitates with

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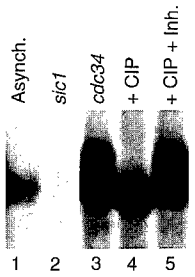

Fig. 1. Sic1 is a phosphoprotein in vivo. Extracts were made as described (*17*), and Sic1 was immunoprecipitated (*14*). The immunoprecipitates were treated or not treated with phosphatase (*18*), resolved by SDS-PAGE (*15*), blotted to nitrocellulose, and Sic1 was detected (*16*). Lane 1, asynchronous cells; lane 2, asynchronous *sic1* cells; lane 3, strain #31 (*19*) arrested at the *cdc34* block at 37°C; lane 4, as in lane 3, but treated with calf intestinal phosphatase (CIP); lane 5, as in lanes 3 and 4, but treated with CIP and the phosphatase inhibitor B-glycerolphosphate (Inh.).

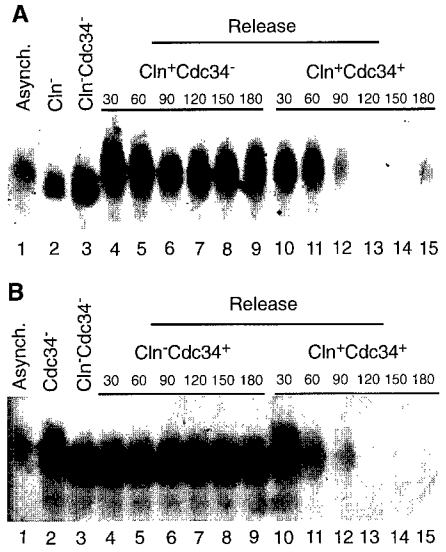

Fig. 2. Loss of Sic1 depends on *CLN*s and on *CDC34*. Abundance and phosphorylation of Sic1 were analyzed in reciprocal shift experiments (*20*). Strain #31 (*cln1 cln2 GAL-CLN3 cdc34*) (*19*) was used. (**A**) Cells were grown in galactose medium at 23°C (lane 1), shifted to glucose at 23°C for 3 hours to synchronize cells at Start (lane 2), then shifted to 37°C for another hour to inactivate Cdc34 (lane 3). Cln expression was then restored by shifting back to galactose medium, but cells were held at 37°C ($Cdc34^-$). Samples were taken every 30 min (lanes 4 to 9). As a control, Cln expression and Cdc34 function were both restored (lanes 10 to 15) to doubly blocked cells. (**B**) Cells were grown in galactose medium at 23°C (lane 1), shifted to 37°C for 3 hours to synchronize cells at the *cdc34* block (lane 2), then shifted to glucose at 37°C for 1 hour to shut off *GAL-CLN3* (lane 3). Cdc34 function was restored by a shift to 23°C, but cells were kept in glucose medium (Cln^-). Samples were taken every 30 min (lanes 4 to 9). As a control, Cdc34 function and Cln expression were both restored (lanes 10 to 15) to doubly blocked cells. FACS analysis showed that the cells in lanes 4 to 9 (A and B) failed to replicate DNA, whereas the cells in lanes 10 to 15 did replicate DNA.

Cdc28 (*4*), has one of the highest densities of potential Cdc28 phosphorylation sites of any known yeast protein (*5*), and can be phosphorylated on many sites by Cdc28 in vitro (*4*, *6*).

Sic1 is a phosphoprotein in vivo. Resolution of Sic1 by SDS–polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting showed a broad, fuzzy band that may contain multiple forms of Sic1. Phosphatase treatment converted this fuzzy band (more phosphorylated form) to a band of greater mobility (less phosphorylated form) (Fig. 1).

To study the relation between the Clns, phosphorylation and proteolysis of Sic1, and DNA synthesis, we constructed a *cln1 cln2 GAL-CLN3 cdc34-2* (temperature-sensitive) strain and did reciprocal shift experiments. As expected, cells shifted from the Cln^-Cdc34^+ state to the Cln^+Cdc34^- state arrested with a $Cdc34^-$ phenotype without dividing. Sic1 accumulated in the less phosphorylated form in Cln^--arrested cells, but was phosphorylated to a greater extent when Cln was restored (*7*) (Fig. 2A, compare lanes 3 and 4). However, in the absence of Cdc34 function (Cln^+Cdc34^-), this highly phosphorylated Sic1 remained undegraded (Fig. 2A, lanes 4 to 9). In control cells arrested in the Cln^-Cdc34^+ state, then released to the Cln^+Cdc34^+ state, Sic1 became more phosphorylated when Cln was restored, and then disappeared, presumably because of proteolysis (*7*) (Fig. 2A, lanes 10 to 15). These cells then reentered a normal cell cycle. Thus, in vivo, the Cln-Cdc28 complexes are needed to generate highly phosphorylated Sic1, which is stable in the absence, but not in the presence, of Cdc34 function.

Cdc34 has been considered to act downstream of Clns and Cdc28. Surprisingly, however, cells shifted from the Cln^+Cdc34^- state to the Cln^-Cdc34^+ state did not enter S phase or divide and in all respects maintained a $Cdc34^-$ phenotype. This result suggests that the Cdc34 function cannot be completed in the absence of Cln-Cdc28 activity. Highly phosphorylated Sic1 accumulated in the Cln^+Cdc34^- cells (*7*) (Fig. 2B, lane 2); Sic1 then became less phosphorylated, but not degraded, after the shift to the Cln^-Cdc34^+ state (*7*) (Fig. 2B, lanes 4 to 9). This result suggests that the $Cdc34^-$ phenotype is maintained in the Cln^-Cdc34^+ cells because the less phosphorylated form of Sic1 cannot be degraded in the absence of Cln activity. When cells were shifted from Cln^+Cdc34^- to Cln^+Cdc34^+, the more phosphorylated form of Sic1 that had accumulated at the *cdc34* block disappeared (Fig. 2B, lanes 10 to 15), and the cells went through S phase and reentered a normal cycle. These experiments show that Sic1 loss requires Cln function as well as Cdc34 function, and that the more phosphorylated form of Sic1 is dependent on Cln activity and correlated with Sic1 loss. Because cells arrest before S phase regardless of the phosphorylation state of Sic1, both forms must inhibit Clb-Cdc28 complexes.

These results are consistent with a model wherein Cln-Cdc28 complexes phosphorylate Sic1, and this phosphorylation targets Sic1 for degradation by the Cdc34 pathway. However, the experiments are correlative, and other mechanisms are also possible. For example, Cln-Cdc28 complexes may serve to activate Cdc34 itself, and the phosphorylation of Sic1 may be a correlated but irrelevant event.

If a major function of Clns is to promote proteolysis of Sic1, then Clns should be less important in a *sic1* mutant. Indeed, a *sic1* mutation suppressed the lethality of a *cln1 cln2 cln3* triple null mutation (Fig. 3B, sectors 1, 3, and 4). Thus, the only nonredundant essential function of the Clns is to inactivate Sic1. The *cln1 cln2 cln3* triple mutation is also suppressed by a mutation

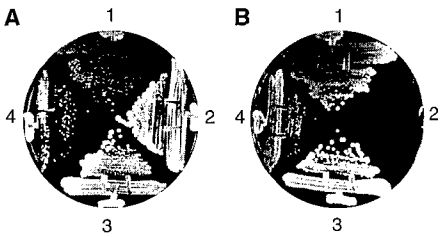

Fig. 3. A *sic1* deletion suppresses lethality of *cln1 cln2 cln3*. (**A**) YEP + 1% raffinose + 1% galactose. (**B**) YEP + 2% glucose. Plates were incubated at 30°C for 3 days. Strains were as follows: 1, BS147 (*pGAL-CLN3 Δclns Δsic1*); 2, BS100 (*GAL-CLN1 Δclns*); 3, BS178 (*GAL-CLN1 Δclns Δsic1*); and 4, BS152 (*Δclns Δsic1*) (*19*).

called *BYC1* (*8*), and it now appears that *BYC1* is allelic to *sic1* (*9*). This suppression by *BYC1* occurs even if *clb2*, *clb5*, or *pcl1* is also deleted (*8*). Clns1, -2, and -3 have other important functions that are compromised in the *cln1 cln2 cln3 sic1* quadruple mutant: Plating efficiency is poor, budding and cell morphology are highly abnormal, and the cells are generally sick. Presumably, budding is now mediated by combinations of other cyclins such as Pcl1, Pcl2, Clb5, and Clb6 (*10*).

If Sic1 is an important and specific inhibitor of replication, then a *sic1* mutation might uncouple DNA replication from other Start events, such as budding. To test this hypothesis, we obtained small unbudded cells from an exponential culture of *sic1* cells and examined the cells for DNA content by fluorescent-activated cell sorting (FACS). At least 20% of the unbudded cells were already 2N, whereas there were essentially no 2N cells in the equivalent fraction from a wild-type culture. After reinoculation into fresh medium, the *sic1* cells

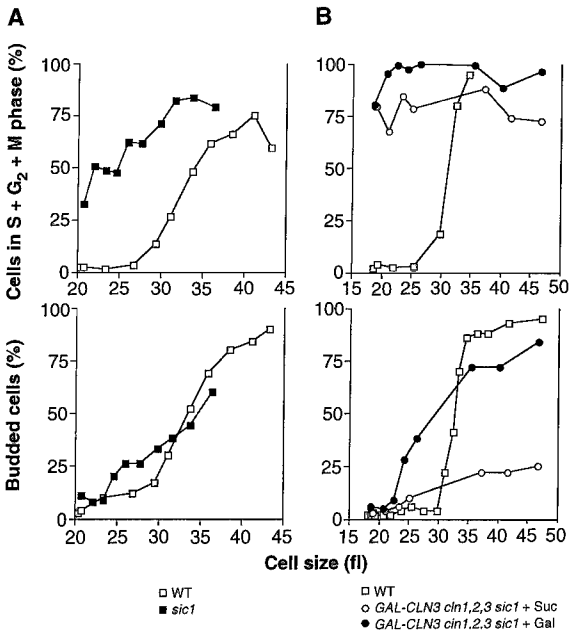

Fig. 4. A *sic1* deletion uncouples S phase from budding. (**A**) Small unbudded cells of strain W303a (*19*) (□) or its isogenic *sic1::URA3* derivative BS193 (■) were obtained by elutriation (*21*). Cells were reinoculated in fresh, warm medium, and samples were taken every 15 min and analyzed for budding, cell volume, and DNA content (FACS) (*22*). (**B**) Strain BS147 (*pGAL-CLN3 Δcln Δsic1*) (*19*) was grown in sucrose plus galactose. Cells were washed and resuspended in medium containing sucrose but no galactose to turn off *GAL-CLN3*. After 1 hour, small unbudded cells were collected by elutriation (*21*). Half the sample was reinoculated into YNB medium with 2% sucrose (*GAL-CLN3* off) (○), and the other half was reinoculated into YNB medium with 1% sucrose and 1% galactose (*GAL-CLN3* on) (●). Samples were taken every 30 min and analyzed as in (A). W303a cells (*19*) grown in YNB + 2% sucrose were elutriated and monitored after reinoculation (□).

replicated DNA much earlier than the wild-type cells, but budded at about the same time (Fig. 4A). [In other, similar experiments, the *sic1* mutation did advance budding slightly, although never as much as the advance in S phase (*2*, *11*). The early activation of Clb5 that occurs in *sic1* cells may advance budding.]

In a second experiment, *cln1 cln2 GAL-CLN3 sic1* cells were grown with *GAL-CLN3* on, and then *GAL-CLN3* was turned off for 1 hour. Small unbudded cells were obtained by elutriation. Fifty to 80% of these cells had a DNA content greater than 1N, despite their lack of Cln. (The large fraction of 2N cells probably resulted from overexpression of *CLB5* induced by *GAL-CLN3*.) When the cells were released into fresh medium, efficient budding was still dependent on reexpression of Cln3, whereas S phase was not (Fig. 4B). Thus, in *sic1* mutants, replication and budding are uncoupled; they occur at different times, and budding is much more dependent on Cln than is replication.

Although phosphorylation and loss of Sic1 are dependent on both Cln and Cdc34 function, we have not shown that Sic1 is a direct substrate of the Cln-Cdc28 kinase in vivo, nor that Sic1 proteolysis is ubiquitin-mediated. However, these are both strong possibilities. Phosphorylation converts at least one other protein into a substrate for Cdc34-mediated proteolysis (*12*). Whatever the precise mechanism by which Clns and Cdc34 cause the loss of Sic1, our genetic experiments show that this loss is largely responsible for the normal dependence of DNA replication on Start.

An analogous system may be used by mammalian cells. Cyclin D–Cdk4 complexes promote S phase by inhibiting function of the retinoblastoma protein. In cells lacking retinoblastoma, the cyclin D–Cdk4 activity is no longer required (*13*).

The identification of Sic1 as a target of Clns suggests that Start consists of several component events. The Start event controlling S phase is probably phosphorylation of Sic1; phosphorylation of other substrates may control budding and duplication of the spindle pole body, and together these phosphorylations constitute Start.

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14. Antibody to rabbit Sic1 (12.5 μl) [J. D. Donovan, J. H. Toyn, A. L. Johnson, L. H. Johnston, *Genes Dev.* **8**, 1640 (1994)] was added to a 3-mg cell extract. After incubation for 1 hour at 0°C, protein A beads (30 μl) were added, and the mixture was rocked at 4°C for 1 hour. Beads were washed four times with alkaline phosphatase buffer (APB) [50 mM tris-HCl (pH 8), 10 mM dithiothreitol, 0.6 mM dimethylaminopurine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (5 μg/ml), tosyl-L-phenylalanine-chloromethyl ketone (10 μg/ml), pepstatin (5 μg/ml), and soybean trypsin inhibitor (10 μg/ml)].
15. Extract (50 μg) was loaded per lane on a 16 cm by 18 cm by 0.75 mm gel and run for 20 hours at 100 V.
16. Proteins were transferred to nitrocellulose for 30 min at 10 V. Blots were blocked by using non-fat milk (5%) in tris-buffered saline [TBS; 140 mM NaCl, 2.5 mM KCl, 25 mM tris-HCl (pH 7.4)] for 1 hour. Blots were incubated overnight with a 1:100 dilution of rabbit antibody to Sic1 (*14*). Blots were washed four times in TBS, then incubated with a 1:2000 dilution of alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G (Pierce) for 1.5 hours, washed, and finally incubated at room temperature with 10 ml of NBT-BCIP (nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolphosphate *p*-toluidine) (Gibco-BRL) for 5 to 10 min. β-Tubulin was used as a loading control.
17. M. Tyers, G. Tokiwa, B. Futcher, *EMBO J.* **12**, 1955 (1993).
18. Beads (30 μl) carrying immunoprecipitated Sic1 (*14*) were divided into three portions (10 μl), and these were treated with APB (10 μl) (*14*), APB (8 μl) plus calf intestinal phosphatase (CIP) (2 μl, 2 U) (Boehringer), or APB (7 μl), CIP (2 μl), and β-glycerolphosphate (1 μl of 1 M).
19. Strains were W303a (*MAT***a** *ade2 his3 leu2 trp1 ura3 can1-100 ssd1-d [psi$^+$]*) [B. J. Thomas and R. Rothstein, *Cell* **56**, 619 (1989)], #31 (*MATα cdc34-2 cln1::HIS3 cln2::TRP1 ura3::GAL-CLN3 leu2 ura3*), BS100 (*MATα cln1::LEU2-GAL-CLN1-HA3 cln2::TRP1 cln3::HIS3 leu2 his3 ura3 ade2 trp1*), BS147 (*MAT***a** *cln1 cln2 cln3 sic1::TRP1 [pGAL-CLN3 CEN URA3] ura3 leu2 trp1 his2 ade1*), BS152 (*cln1 cln2 cln3 sic1::TRP1*) (derived from BS147 by plasmid loss), and BS178 [(*MAT***a** *cln1::LEU2-GAL-CLN1-HA3 cln2 cln3 sic1::TRP1 ura3 trp1 his*(2 or 3)]. The *sic1::TRP1* allele was from M. Tyers; the parent of BS147 was from F. Cross.
20. Cells were grown to 1×10^7 cells per milliliter. The galactose medium was YEP (1% yeast extract, 1% peptone) with 1% raffinose and 1% galactose; the glucose medium was YEP with 1% raffinose and 2% glucose. Before shifting from one medium to another, cells were first washed twice with the new medium that had been prewarmed to the target temperature. Sic1 was detected as described (*15*, *16*). Representative samples were treated with phosphatase as shown (Fig. 1) to demonstrate that the mobility shift was due to phosphorylation.
21. Cells were grown in SD medium [F. Sherman, *Methods Enzymol.* **194**, 3 (1991)] with required amino acids to 2×10^7 cells per milliliter with 2% filter-sterilized sucrose (W303 and BS193) or 1% filter-sterilized sucrose plus 1% galactose (BS147). Cells were centrifuged, sonicated, and elutriated in medium at 30°C.
22. A computer curve-fitting algorithm estimated the number of cells with DNA content of 1N, 2N, or between 1N and 2N.

23. We thank L. Johnston for antibody to Sic1; M. Mendenhall, M. Tyers (who independently found suppression of the *cln1 cln2 cln3* mutant by *sic1*), and F. Cross for strains, reagents, helpful discussions, and communication of unpublished results; and M. Cleary and M. Luke for reading the manuscript. Supported by NIH grant GM 39978 and U.S. Army Breast Cancer grant DAMD17-94-J-4050 to A.B.F.

11 December 1995; accepted 28 February 1996